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RNAi Inhibition of Rabbit Hemorrhagic Disease Virus (RHDV) Gene Expression in CHO Cell Line

استعمال (RNAi) من اجل تثبيط التعبير الجيني لفيروس النزف عند الأرنب باستخدام خلايا مبيض الفأر الصيني (CHO)

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Abstract

Rabbit hemorrhagic disease virus (RHDV) causes great losses and is life threatening to both wild and domestic rabbits. This work represents the first step toward production of transgenic rabbits resistant to RHDV by RNAi through the inhibition of expression of a part of the RHDV genome and the consequent inhibition of its multiplication. This part of RHDV genome is available under the accession EU003582.1 in NCBI from the nucleotide 372 to 392 (McIntosh *et al.*, 2007). Chinese Hamster Ovary (CHO) cell line was transfected with a plasmid harboring the RHDV target in the DNA segment coding for luciferase (fusion protein) and co-transfected with another plasmid harboring a DNA sequence designed to code for a homologous double stranded RNA sequence of 21 pb. This sequence acts as siRNA to inhibit the expression of the viral genomic part through RNAi. The series of co-transfection resulted in an inhibition level ranging from 82 to 87% of expression of the fusion protein and consequently of the RHDV target. These results are encouraging. However, further investigations are necessary to obtain more inhibition *ex-vivo* with validation *in vivo* using transgenic rabbits.

Key words: RNA interference (RNAi), small interfering RNAs (siRNAs), microRNA (miRNA), rabbit hemorrhagic disease virus (RHDV), regulation of gene expression by silencing, CHO cell line, transgenic animals.

ملخص

يسبب فيروس النزف عند الأرنب (RHDV) خسارة فادحة في قطاع التربية وفي الحيوانات البرية أيضا. يمثل هذا العمل الخطوة الأولى باتجاه إنتاج أرانب مقاومة لهذا الفيروس باستخدام تقنية مبنية على ظاهرة تداخل الحمض النووي الريبوزي مزدوج السلسلة (RNAinterference) من خلال تثبيط التعبير الجيني لجزء من منظومة الفيروس الوراثية وبالتالي منع تكاثره. هذا الجزء متاح تحت الرقم EU003582.1 في الـ NCBI من النيوكليوتيدة ٣٧٢ وحتى ٣٩٢ (McIntosh *et al.*, 2007). تم إدخال بلازميد في خلايا مبيض الفأر الصيني، يحتوي هذا البلازميد بداخل جين اللوسيفيريز على جزء من منظومة الفيروس الوراثية (بروتين التحامي)، وتم كذلك في نفس الخلايا إدخال بلازميد آخر يحتوي على DNA مصمم بهدف أن يشفر RNA الذي ينطوي على نفسه ليكوِّن سلسلة RNA مزدوج السلسلة بطول ٢١ زوجا من النيوكليوتيدات. هذه السلسلة مصممة لتعمل ك siRNA وتثبط بواسطة ظاهرة تداخل الـRNA التعبير للجزء المذكور أعلاه من منظومة الفيروس المتطابقة من حيث السلسلة النيوكليوتيدية (RNAi). أظهرت النتائج تثبيطا للتعبير الجيني الفيروسي ممثلا بقراءات البروتين الإلتحامي (اللوسيفيريز) يتراوح ما بين ٨٢ إلى ٨٧% من التعبير للجين الالتحامي وبالتالي لذلك الجزء من منظومة الفيروس بداخله. هذه النتائج مشجعة إلا أنها تحتاج إلى مزيد من الأبحاث لإقرارها وللحصول على مزيد من التثبيط باستخدام خلايا ومن ثم باستخدام أرانب معدلة وراثياً.

Introduction

Rabbit Hemorrhagic Disease (RHD) is a highly contagious, severe acute viral illness. Typically, the disease presents with fever and sudden death (Marcato *et al.,* 1991; Capucci *et al.,* 1991). Rabbits will often develop a blood-tinged foamy nasal discharge, severe respiratory distress and/or convulsions preceding death. Mortality rates are high, ranging from 70% to 95% (reviewed by McIntosh, 2007).

The RHDV genome is a ssRNA genome with no DNA stage. It is composed of 7447 nucleotides (McIntosh *et al.,* 2007). The following proteins are coded by this genome: p16, p23, and p37 nonstructural proteins, VPg, a putative nonstructural protein of approximately 30 kDa,

a trypsin-like cysteine protease of 15 kDa, a putative RNA polymerase of 58 kDa, the major capsid protein VP60 and a minor structural protein of 10 kDa (Wirblich *et al.,* 1996).

RNAi was first discovered in the petunia plant (Napoli *et al.,* 1993). Later on, RNAi was found to be involved in the regulation of several genes that control the plant's shape in *Arabidpsis* (Palatnik *et al.,* 2003). RNAi was also described for the worm *Caenorhabditis elegans* (Lee *et al.* 1993).

Plants harboring only short sequences of the viral RNA genome produced tolerance or resistance against the corresponding viruses (Stram and Kuzntzova, 2006). Short fragments of plant gene sequences were introduced into plant viruses which were used to infect plants. The expression of the targeted plant gene was suppressed after infection of plants with the modified viruses (Metzlaff *et al.,* 1997)

The causative agent of the RNAi was identified to be the double stranded RNA. Although the full mechanism is not yet fully elucidated, the ribonuclease enzyme appropriately called Dicer interacts with the dsRNA and cleaves it into short double stranded RNA molecules of 21 to 23 bp. The structural basis for double-stranded RNA processing by Dicer is described by Macrae *et al.,* 2006. The short ds fragments resulting from Dicer action are called small interfering RNAs (siRNAs). These ds siRNAs are split off into single stranded RNA molecules (ssRNA). The resulting ssRNA molecules are integrated into a complex of multiproteins called the RNA-induced silencing complex (RISC), which cleaves the mRNA (Lodish *et al.,* 2004). In *Caenorhabditis elegans*, which is widely used in the RNAi research (Dzitoyeva *et al.,* 2003), an effective RNAi response requires the production of secondary siRNAs by RNA-directed RNA polymerases (RdRPs). The RdRPs perform unprimed RNA synthesis (Sijen *et al.*, 2007). The nature of the populations of siRNA molecules involved in RNAi was investigated in details by Pak and Fire, 2007. RNAi has a systemic and generally a heritable effect in plants as well as in *C. elegans*. RNAi takes place in plants via the movement of siRNAs through plasmodesmata (Lodish *et al.,* 2004).

In bacteria, genes that control mRNA abundance or translation by binding to matching mRNA molecules were identified, but are not considered to be siRNAs or miRNA's because the Dicer enzyme is not involved (Morita *et al.,* 2006).

The first application of RNAi to reach clinical trials is in the treatment of macular degeneration. RNAi has also been shown effective in the complete reversal of induced liver failure in mouse models (Zender *et al.*, 2003) and in silencing of hepatitis A virus infection (Kusov *et al.,* 2006). A retrovirus-based system to stably silence hepatitis B virus genes by RNA interference is described by Jia, 2006. Encouraging results were obtained for the inhibition of propagation of influenza virus (Li, 2005) and for inhibition of Measles virus multiplication in cell culture (Hu, 2005). RNAi could also be used to silence the expression of some of the genes required for infection by the majority of primary HIV-1 isolates (Lee, *et al.,* 2003). Experimental cancer therapy by siRNAs can open a treatment horizon in the future (Tong, 2005). The therapeutic potential of RNAi for neurological disorders makes it promising (Sah, 2006).

Objective of this Study

The need for an efficient prevention against RHDV is of vital importance for rabbits in domestication and the wild. Although an efficient vaccine (a recombinant protein to be injected into the young rabbits) was prepared by INRA (France) to protect against infection with RHDV, its use is, however, problematic and restricted to domestic animals. Considering all the reported results of the RNAi phenomenon, it was appealing to think about protecting rabbits against this disease using RNAi. In this paper, RNAi will be assayed for inhibiting the expression of a part of the genome of RHDV with the horizon of creating transgenic rabbits resistant to RHDV. An RNAi special construct will, therefore, be used to suppress the expression of a part of the RHDV viral genome (inserted in luciferase as a fusion protein) through the co-transfection both constructs in the CHO cell line, and then assaying for the luciferase activity as an indicator of the suppression level by RNAi.

Methodology

Design of the RHDV RNAi seq (RNAi construct)

RHDV RNAi seq (RNAi construct) was constructed in two steps using pBS/U6 (figure 1). In the first step, the pair of oligos RHDV1a/RHDV1b (ggcattgaacaaggttatccca and ccgtaacttgttccaatagggttcga respectively) was used followed by the pair of oligos RHDV2a/RHDV2b (agcttgggataaccttgttcaatgcccttttttg and accctattggaacaagttacgggaaaaacttaa). RHDV1a/RHDV1b are designed to have (upon annealing) a blunt end on one extremity and a *Hind*III compatible end on the other extremity. RHDV2a/RHDV2b are designed to have (upon annealing) a *Hind*III compatible on one end and a blunt end on the other extremity. After cloning the mentioned two pairs of oligos, the double stranded DNA fragment cloned downstream U6 promoter called hence for RHDV RNAi seq (RNAi construct) should code (upon transcription) for a single stranded RNA molecule. This RNA molecule is designed to fold upon itself to form the following 21 double stranded RNA molecule

ggcauugaacaagguuauccc

ccguaacuuguuccaauaggg

This double stranded RNA molecule is homologous to a part of the RHDV genome that was cloned in pcDNAMLC. This part is available under the accession EU003582.1 in NCBI (McIntosh *et al.,* 2007) from the nucleotide 372 to 392 (the entire RHDV genomic sequence is composed of 7447 nucleotides). This part is a component of an expressed sequence coding for a polyprotein (McIntosh *et al.,* 2007) which is available in NCBI (protein_id ABV56618.1). For simplicity, this part of the RHDV genome will be named RHDV target throughout this paper. RHDV target was chosen on the basis of the possibility of use of RHDV RNAi seq (RNAi construct) to create transgenic rabbits (in future investigations). Therefore, in order to avoid possible silencing of essential rabbit endogenous genes, a search on BLAST was performed to avoid common sequences with the rabbit genome. The chosen RHDV target fills this requirement.

Cloning of the RHDV RNAi seq (RNAi Construct) in pBS/U6 plasmid

A pair of oligos RHDV1a/RHDV1b (ggcattgaacaaggttatccca and ccgtaacttgttccaatagggttcga respectively) were annealed and cloned in the plasmid pBS/U6 down stream of the promoter U6. The pBS/U6 plasmid was supplied by Prof. L. M. Houdebine, INRA, France (figure 1). Cloning was accomplished by cutting pBS/U6 with the restriction enzyme *Apa*I (Biolabs) at RT overnight, and then removing the 3' overhang and filling the 5' overhangs using T4 DNA polymerase (Biolabs) to form a blunt end (Tobor S. and Struhl K., 1989). Then the processed pBS/U6 was digested with *Hind*III (Biolabs) for 3 hrs at 37°C and then purified by phenol/chloroform twice, and then the cut plasmid was precipitated by Na acetate and ethanol (Sambrook *et al.*, 1989). The pair of RNA oligos (RHDV1a/RHDV1b) was boiled in water for 5 minutes then was left to anneal at RT for 30 minutes. Upon annealing this pair of oligos gives a double stranded DNA molecules possessing a blunt end and a *Hind*III cohesive end. This annealed fragment was inserted downstream of the U6 promoter in the prepared pBS/U6 plasmid in a 1 to 30 vector to insert ratio. A rapid ligation kit supplied by Roche Applied Science was used according to the manufacturer's instructions. In a second step, RHDV2a/RHDV2b were left to anneal. They are designed to have *Hind*III and *EcoR*I ends upon annealing. The annealed fragment was cloned (in the same manner) down stream of the first annealed sequence after digesting the plasmid harboring it with *Hind*III and *EcoR*I (figure 1). Transformation was performed into the competent cells DH5α.

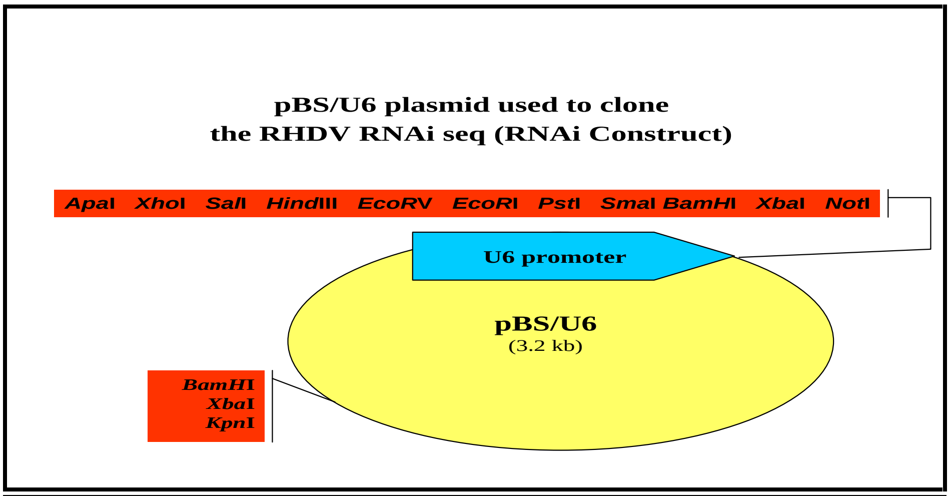


Figure (1): pBS/U6 plasmid used to clone the RHDV RNAi seq (RNAi Construct).

Screening for the recombinant colonies was also accomplished in two steps using Southern blotting on Hybond N+ membranes (Amersham) following the digestion of the bacterial plasmid DNA with *BamH*I, and hybridization with a probe of RHDV1b (ccgtaacttgttccaatagg) to screen for the pBS/U6 harboring the first annealed fragment (RHDV1a/RHDV1b) and the probe of RHDV2a (agcttgggataaccttgttcaatgcccttttg) to screen for the recombinant plasmid harboring the second annealed fragment (RHDV2a/RHDV2b) (figure 1). The pre-hybridization took 3 hrs at 50°C and hybridization was performed over 72 hrs at 50°C. The first screening gave results shown in figure 2-a. The A42 clone was taken for the second cloning and screening gave results shown in figure 2-b where α3 gives a promising signal. α3 was, therefore, sequenced and proved to contain both pairs of oligos (RHDV1a/RHDV1b upstream of the pair of oligos RHDV2a/RHDV2b). α3 was therefore the suitable recombinant and was kept for the subsequent RNAi work. The resultant double stranded RNA molecule of 21 bp coded by α3 was designed to be compatible with a target sequence in the genome of RHDV cloned in the vector pcDNAMLC supplied by Prof. L. M. Houdebine, INRA, France (Figure 3).

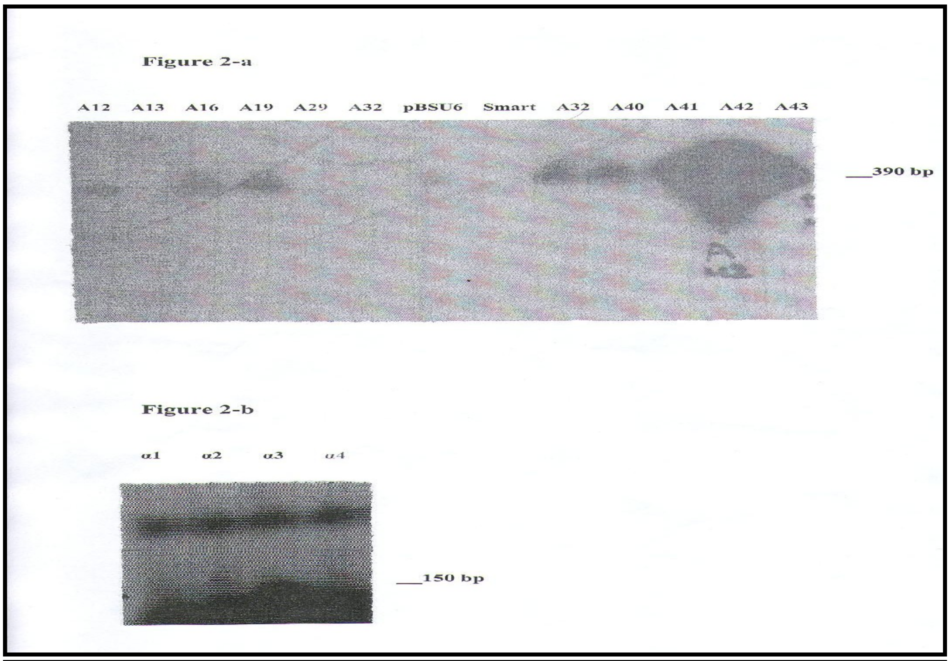


Figure (2): ***(a)*** This Southern blotting autoradiogram was used to determine the plasmid pBS/U6 harboring the first annealed DNA fragment (RHDV1a/RHDV1b). Plasmids were digested with *BamH*I (see figure 1) according to the manufacture’s instructions (NEB), the gel was transferred on Hybond N+ membranes (Amersham) and hybridized with a probe of RHDV1b (ccgtaacttgttccaatagg). The positive clones give signals on the band of approximately 390 bp. These clones are A41, A42 and A43. Smart is the molecular size ladder used

(b) This autoradiogram of Southern blotting was used to determine the recombinant plasmid issued in figure 2-a harboring the second annealed DNA fragment (RHDV2a/RHDV2b). The plasmid was digested with *BssH*II and *Sac*I (see figure 1) according to the manufacture’s instructions (NEB). The gel was transferred on Hybond N+ membranes (Amersham) and hybridized with a probe of RHDV2b. The positive clones give signals on the band of approximately 150 bp. Clone α3 seems to be promising.

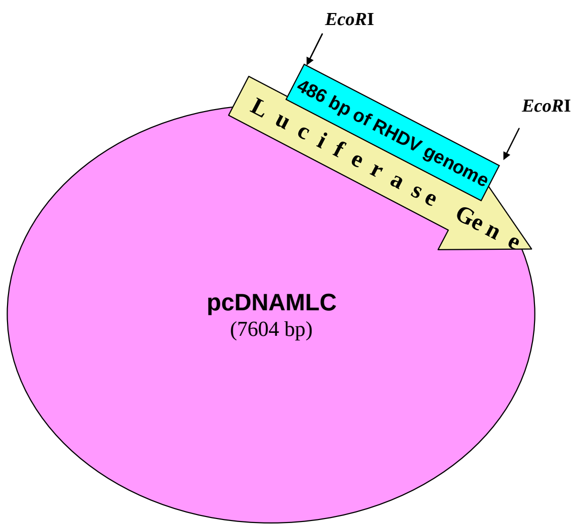


Figure (3): pcDNAMLC vector harboring 486 bp of the RHDV target. This RHDV fragment starts at the nucleotide position 79 and ends at the nucleotide position 564 in the sequence available under the accession EU003582.1, NCBI (McIntosh *et al.*, 2007). This fragment from the viral genome was cloned in the restriction site of *EcoR*I in the luciferase gene of which expression was maintained (fusion protein). pcDNAMLC vector was kindly provided by Prof. L. M. Houdebine, INRA, France. The gene luciferase starts 6 nucleotides before the fragment of RHDV and ends 249 nucleotides after it.

Labeling

The labeling was performed through transfer of ^{32}P from ATP (3^{rd} position) to the 5' end of RHDV1b sequence using T4 kinase for

screening for pBS/U6 harboring the annealed DNA fragment RHDV1a/RHDV1b. RHDV2b was labeled in the same manner for screening of the latter plasmid harboring the annealed DNA fragment RHDV2a/RHDV2b. 3 µl of ^{32}P (10 m Ci/ml) purchased from Amersham^{TR} were mixed with 6 pmol of RHDV1b or RHDV2b, 1µl of kinase buffer 10X and 10 units of kinase (Eurogentec) in a final volume of 10 µl (complemented with distilled water). Reaction was conducted at 37 C during 30 min then kinase was inactivated by incubating the reaction tube at 65 C/5 min.

Transfection and Assaying for Luciferase Activity

The RHDV viral part cloned in luciferase gene (fusion protein) was co-transfected with the RNAi construct in CHO cells. The decrease in the expression of the fusion protein (luciferase) reflects the inhibitory effect of the RNAi construct *ex-vivo* (in CHO cells).

TKLuc plasmid was used in experiments as a reference of strong activity of the luciferase reporter gene. Basic TKluc reporter vector was constructed as follows: A *BamH*I-*Xba*I fragment, containing the Herpes Simplex Virus (HSV) Thymidine Kinase (TK) promoter, was obtained from pT109luc plasmid (Nordeen, 1988) and inserted into pGL2basic luciferase reporter vector (Promega), cleaved with *Bgl*II-*Xba*I (Figure 4)

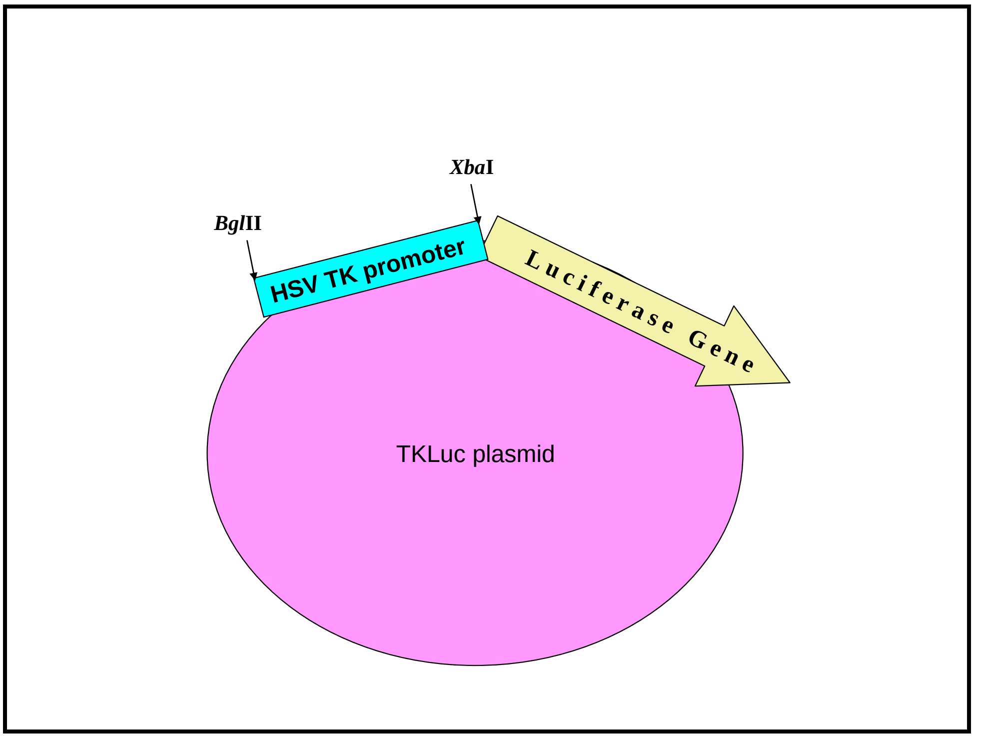


Figure (4): TKLuc plasmid: A *BamH*I-*Xba*I fragment, containing the Herpes Simplex Virus (HSV) Thymidine Kinase (TK) promoter, was inserted into pGL2basic luciferase reporter vector (Promega), cleaved with *Bgl*II-*Xba*I (BamHI cut end is compatible with *Bgl* II cut one).

Lipofectamine reagent purchased from Invitrogen Life Technologies was used for the co-transfection experiments according to the manufacturer's recommendations with minor modifications. The complex DNA-Lipofectamine in Calf Fetal (CFS) and Penicillin, Streptomycin and Gentamycine (PSG) free medium was added to 95% confluent Chinese Hamster Ovary (CHO) cultures. The cultures were incubated at 37°C for 19 hrs, then 12 ml of full medium with CFS (10%) and PSG (Penicillin (100 units/ml), Streptomycin (100 µg/ml) and Glutamine (2 mM) were added per 60 mm Petri dish. Collection and lyses of cultures were performed 46 hrs after the beginning of transfection, cells of each

Petri dish were collected in 100 µl of lysis buffer (glycyl glycerine 25 mM, EDTA 1 mM, $MgSO_4$ 8 mM, Triton 1%, glycerol 15% and DTT 1 mM added extemporarily) and immediately 10 µl of lysed cells were added to 45 µl of luciferase substrate and inserted immediately into the luminometer. Readings of luciferase were taken, they reflect the capacity of the RNAi construct to inactivate RHDV target harbored in the luciferase gene (fusion protein construct).

Two series of co-transfection experiments were conducted in order to evaluate the capacity of the designed dsRNA (RNAi construct) molecules in inhibiting the expression of luciferase (fusion protein); each co-transfection was composed of 3 replicates. The target sequence plasmid (pcDNAMLC) was co-transfected with the RHDV RNAi seq (RNAi Construct) in the CHO cell line.

Statistical Analysis

Error limits cited and error bars plotted represent simple standard deviations of the mean. Usually, numerical results are only accurate enough to specify the least significant digit. When comparing different samples, results were considered to be statistically different when $P<0.05$ (Student's t-test for unpaired samples).

Results and Discussion

The first series of experiments was performed to evaluate the capacity of the designed dsRNA molecules to inhibit the expression of luciferase. The target sequence, a part of the genome of RHDV was inserted in the luciferase expression sequence in the plasmid pcDNAMLC. Luciferase maintained, however, its expression as a fusion protein. The experiment was to investigate the capacity of RNAi produced by the dsRNA to inhibit the expression of luciferase and consequently the expression of RHDV target. The co-transfection experiments gave results in the form of the activity of luciferase (Fig. 5).

Bars in figure 5 (and also in figure 6) show the readings of luciferase activity. These readings reflect the capacity of the RNAi construct to

inactivate the luciferase gene harboring part of the RHDV genome (RHDV target). The exact values can be retrieved from the figure using Prism^{TM} package.

Upon co-transfection of α3 with pcDNAMLC (harboring the RHDV target sequence for RNAi seq), a clear inhibitory effect of 82% on the expression of luciferase (as a fusion protein) was demonstrated (left bar). This inhibitory effect is in comparison with the virgin plasmid (pBS/U6) and therefore is specific (figure 5).

To demonstrate furthermore the specificity based on homology between the RNAi contructs and their target gene to be inhibited, we can see that U6β (which harbors a sequence RNAi for another virus, the Aujesky virus) has no inhibitory effect on pcDNAMLC. The expression level of cotransfection of pcDNAMLC+ U6β is similar to pcDNAMLC+ pBS/U6 which is the initial plasmid harboring no RNAi constructs. Therefore, it seems that only the homologous RNAi constructs have a specific inhibitory effect on the expression of luciferase gene. Taken together, the RNAi construct designed in this study (α3) has strong and specific inhibitory effect on the expression of the corresponding homologous target sequence (figure 5). The expression of luciferase was specifically inhibited and not vulnerable to a non-homologous RNAi construct (U6β). For inhibition of expression, homology seems, therefore, to be essential between the target and the RNAi construct.

TKLuc is known to have a very strong expression level of luciferase. It was co-transfected with pBS/U6 (left bar) and also co-transfected with α3 which is pBS/U6 harboring the RHDV RNAi sequence (next bar). For clarity, the luciferase readings were divided by 10. Figure 5 demonstrates that α3 has no clear inhibitory effect on the expression of luciferase in TKLuc compared with pBS/U6. In other words, the very high level of expression of TKLuc was not clearly influenced by α3 compared with the initial plasmid (pBS/U6). So, there was no clear effect of the RHDV RNAi seq on luciferase expression when there is no homology between the two sequences.



Figure (5): Readings of luciferase activities in the CHO lysates after cotransfection of the shown pairs of plasmids. α3 (RHDV RNAi seq or RNAi Construct) has a clear specific inhibitor effect of 82 % on the expression of luciferase gene harboring the target homologous sequence.

These results were confirmed by another series of experiments, each composed of 3 replicates of co-transfections (Fig. 6) with an additional plasmid, pcDNAMLC3. The later plasmid possesses a strong expression level of luciferase. It was co-transfected with pBS/U6 as well as with α3 which is pBS/U6 harboring the RHDV RNAi sequence (on left). α3 has no clear inhibitory effect on the expression of luciferase in comparison with the initial plasmid pBS/U6. But as the right bars in figure 6 show, α3 leads to a decrease of 87% in the activity of luciferase (fusion protein

construct) harboring an RHDV fragment of genome identical to the RNAi construct in α3 (pcDNAMLC) in comparison with pBS/U6 which is the initial plasmid. This demonstrates a strong inhibitory effect of RHDV RNAi seq. on the expression of luciferase gene harboring a matching sequence of RNAi seq.

As noted, U6β harbors a sequence of RNAi for another virus (Aujesky virus). When the expression profiles of pcDNAMLC+U6β and pcDNAMLC+ α3 are compared, we see that α3 has a clear inhibitory effect on the expression of luciferase gene harboring the homologous RHDV target (figure 6). It is clear from the middle bar in figure 6 that the U6β RNAi construct again has no effect on the expression of the luciferase gene which does not harbor any target homologous sequence. The effect is similar to the initial plasmid (pBS/U6).

Results obtained in figures 5 and 6 demonstrate a specific inhibition which is conditioned by complementarity between the target and the RNAi construct. They open promising horizons for an efficient genetic combat against the rabbit hemorrhagic disease virus (RHDV).

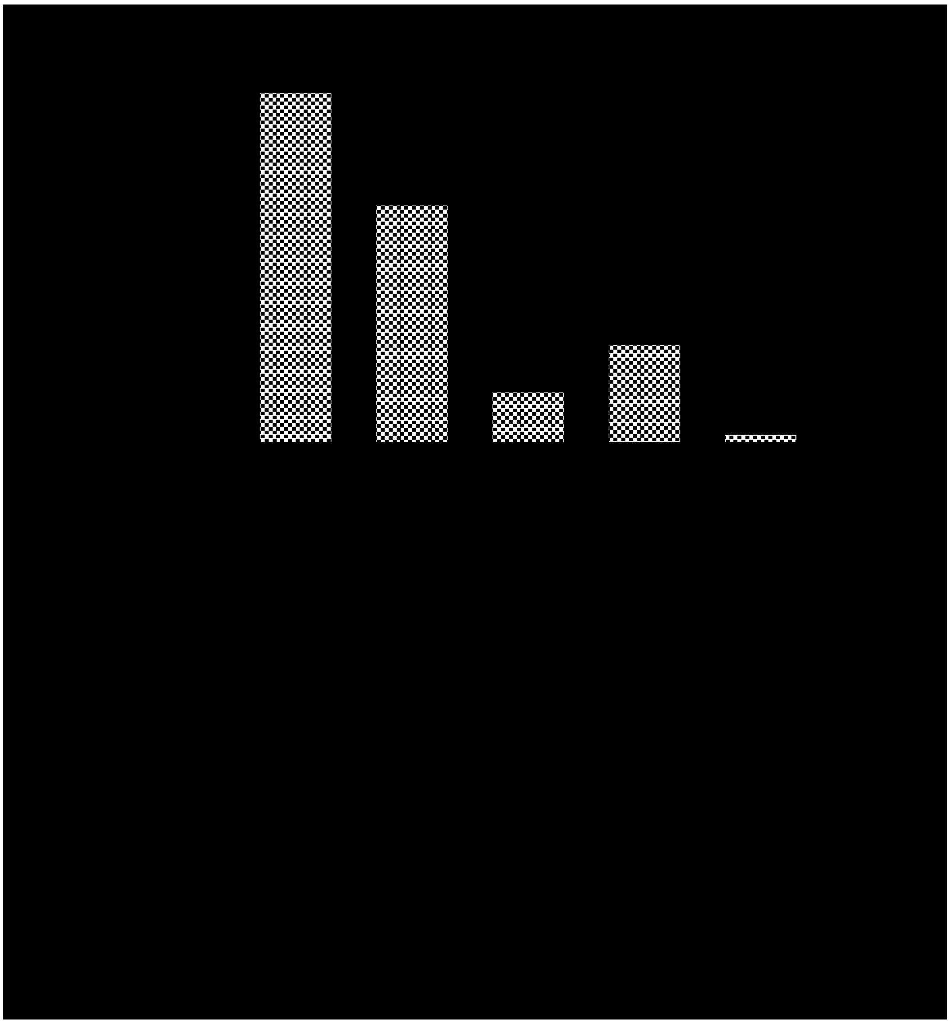


Figure (6): Luciferase activities in the CHO lysates after co-transfection of the shown pairs of plasmids. α3 (RHDV RNAi seq or RNAi Construct) has a clear specific inhibitor effect (87%) on the expression of luciferase gene harboring the target homologous sequence. The inhibition is conditioned by homology between the RNAi construct and the target sequence (see Results and Discussion). The luciferase readings were so strong that they were divided by 50 for clarity.

In this study, a variation of expression inhibition (from 82 to 87%) by RNAi was demonstrated. In other studies, variation in RNAi inhibition of gene expression is also encountered. For example, using RNAi, fgf8 gene expression inhibition varied from 0 to 100% (Hernandez-Hernandez, 2001).

It has been demonstrated by many groups that RNA interference, induced by transfection of small interfering RNA (siRNA) duplexes, can protect cells against a viral challenge in culture, this protection is, as in this study, transient. However, it was demonstrated that lentivirus expression vectors can stably express siRNAs at levels sufficient to block viral replication. These vectors stably express siRNAs specific for HIV-1 *Tat* transcription factor or specific for a cellular coreceptor (CCR5) that is required for infection by the majority of primary HIV-1 isolates (Lee *et al.*, 2003).

Finally, techniques based on the powerful phenomenon of RNAi can be extrapolated for other application in health and agriculture in the future (e.g. it could be applied on other viral diseases and other animal, human and plant pathogens).

Conclusions

In this study an RNAi construct was used to inhibit the expression of the RHDV target. Inhibition varied between 82 and 87% and was specific and conditioned by homology between the RNAi construct and the target gene to be inhibited. The importance of this research is clear from an applied as well as fundamental point of view. It provides the basis for further investigations aiming to create transgenic rabbit resistant to the infection by RHDV. The technique of RNAi is sometimes referred to as a "knockdown" since RNAi may not totally abolish expression of the gene, to distinguish it from "knockout" procedures where a complete elimination of expression is obtained by knocking out or removal of the corresponding DNA fragment (Zhao, 2005). Nevertheless, the RNAi technique has a strong potential to help understanding the function of genes through specific silencing of genes under demand.

Perspectives

This study represents a basis for an efficient combat against RHDV in rabbits. Further investigations will be valuable for confirming results in CHO and other types of cells. In another study, it was shown that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney and HeLa cells (Elbashir, 2001). Other RNAi constructs targeting other viral parts of RHDV can give more inhibition. The most efficient RNAi construct could be used ultimately in transgenic animals to obtain genetically resistant rabbits against RHDV. Viral genomic data are available and can be used to achieve such perspectives (McIntosh *et al.,* 2007).

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